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⑮ 発明の名称 新規なペプチド及びアンジオテンシン変換酵素阻害剤

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明 細 書

1. 発明の名称 新規なペプチド及びアンジオテンシン変換酵素阻害剤

2. 特許請求の範囲

1) 下記の式

Leu-Lys-Pro

で表されるペプチドおよびその塩。

2) 下記の式

Leu-Lys-Pro

で表されるペプチドまたはその塩を有効成分とするアンジオテンシン変換酵素阻害剤。

3. 発明の詳細な説明

〔産業上の利用分野〕

本発明は新規なペプチドおよび該ペプチドまたはその塩を有効成分とするアンジオテンシン変換酵素阻害剤に関する。

〔従来の技術〕

血圧上昇をもたらす代表的な生体内因子としてレニン・アンジオテンシン系が、また血圧降下に因る代表的な生体内因子としてカリクレ

イン・キニン系が知られているが、アンジオテンシン変換酵素(以後「ACE」という)はこのいずれの系にも大きく関与している。

この機構を簡単に説明する。まず、レニン・アンジオテンシン系では、血中に分泌された腎臓の酵素レニンが血中のアンジオテンシノーゲンに作用してテトラペプチドであるアンジオテンシンIを生成する。このアンジオテンシンIは血圧上昇作用を示さないが、これにACEが作用するとオクタペプチドであるアンジオテンシンIIを生成する。このアンジオテンシンIIは末梢血管を収縮させるとともに、副腎皮質に作用してアルドステロンの産生を促し、アルドステロンは腎臓に作用してナトリウムの再吸収、尿量増加を用いて心拍出量の増大をもたらす。そのいずれもが血圧を大きく上昇させる。

一方、カリクレイン・キニン系では血中の酵素タンパク質であるキニンノーゲンに血中酵素のカリクレインが作用してキニンを遊離放出するが、このキニンは末梢血管を拡張させると

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もにホスホリパーゼA₂を活性化してプロスタグランジンの合成を促進して血圧を低下させる。ところがこのカリクレイン・キニン系にACEが働くと、ACEは末梢血管の拡張作用およびホスホリパーゼA₂の活性化作用を有する上記キニンを分解・不活性化してしまうために、血圧の降下が生じなくなる。

したがって、ACEの上記のような働きを阻害する物質(ACE阻害剤)が存在すると、血圧上昇物質であるアンジオテンジンの生成が抑制され、且つ血圧降下物質として働くキニンの分解が防止されて、血圧の上昇抑制および血圧降下が可能になる。

かかる点から近年ACE阻害剤の研究開発が盛んに行われており、天然タンパク質由来の、または合成による特定のペプチド鎖がACE阻害作用を有することが報告されている。これまでに特許された天然タンパク質由来のACE阻害ペプチドとしては、マムシ由来のブラジキニン・ポテンシエーターC(Pyr-Gly-Leu-Pro-Pro

-Arg-Pro-Lys-Ile-Pro-Pro)およびブラジキニン・ポテンシエーターC(Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro)(いずれもH. Kato and T. Suzuki, Biochemistry, 10, p.972 (1971)に記載されている)、牛乳カゼイン由来のペプチドであるPho-Phe-Val-Ile-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys(特公第60-23025号公報)、Pho-Phe-Val-Ile-Pro(特開第59-44323号公報)、Thr-Thr-Met-Pro-Leu-Tyr(特開平3-20283号公報)、Ala-Val-Pro-Tyr-Pro-Gln-Arg、魚類タンパク質由来のペプチドであるTyr-Lys-Ser-Phe-Ile-Lys-Gly-Tyr-Pro-Val-Met、Pro-Glu-Glu-Glu-Pro-His-Val-Leu、トウモロコシアーゼイン由来のペプチドであるLeu-Pro-Pro-Val-His-Leu-Pro-Pro、Val-His-Leu-Pro-Pro-Pro等を挙げることであるが、その大半はアミノ酸が5個以上重合したペプチドである。

(発明の要旨)

上記のような状況下には本発明者らもACE阻害作用を有する物質について研究を進めてきた。

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その結果、上記既知のACE阻害ペプチドとは異なるアミノ酸配列を有する、ロイシン-リジン-プロリンが配列した新たなトリペプチドLeu-Lys-Proを乳清タンパク質の加水分解物から単離することができ、そしてこのトリペプチドがACE阻害作用を有することを見出した。

したがって、本発明は、下記の式



で表されるペプチドおよびその塩である。

更に、本発明は上記式で表されるペプチドまたはその塩を有効成分とするACE阻害剤を含む。

本発明の上記ACE阻害剤を有するペプチドは、最初乳清タンパク質のプロテアーゼによる加水分解生成物として発見されたものであり、その場合には上記3個のアミノ酸Leu、LysおよびProはいずれもL-アミノ酸である。しかしながら、それに限定されず上記のアミノ酸配列を有するトリペプチドであればいずれの

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アミノ酸配列であってもよく、23個のアミノ酸の全部がD-アミノ酸からなるトリペプチドおよび3個のアミノ酸のうちいずれか1つまたは2つがL-アミノ酸であって残りがD-アミノ酸からなるトリペプチドも包含され、それらは化学合成により製造することができる。

本発明のトリペプチドの調製法の例を挙げると以下のとおりである。

乳清タンパク質の加水分解による方法

乳清タンパク質をプロテアーゼを使用して加水分解して水溶性の乳清タンパク質由来ペプチド混合物を調整する。その間に、乳清タンパク質を水等の液体中に分散または溶解させた状態で加水分解を行うのが、操作のし易さ、目的物の収量の確保の点から好ましい。

プロテアーゼとしては、酸性で作用するプロテアーゼ、特に終末の活性中心にアスパラギン残基とアスパラギン側のカルボン酸イオンが関与するアスパルティックプロテアーゼを用いるのがよい。そのようなプロテアーゼの例

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としては、ペプシン、ヒドロゲン酸のアスパーテックプロテイナーゼ、アスベルギス菌のアスパーテックプロテイナーゼ、ペニシリウム菌のアスパーテックプロテイナーゼを挙げることができる。特にペプシン、アスベルギス菌のアスパーテックプロテイナーゼが目的物を高収率で得ることができる点で好ましい。プロテアーゼは1瓶瓶のみを使用しても、またはプロテアーゼ同士がお互いに相影響を及ぼさないかぎり複数種を併用してもよい。複数のプロテアーゼを使用する場合は、複数のプロテアーゼを同時に存在させて加水分解を行っても、または1種ずつ逐次に用いて加水分解を行ってもよい。また、プロテアーゼはフリーの状態で使用しても、固定化して使用してもよい。プロテアーゼの使用量はいずれの場合も乾燥したグルテン100g当たりプロテアーゼ約5,000~100,000 unitsを用いるのがよい。

ここで本明細書中のプロテアーゼ活性(unit)はすべて下記の方法により測定したものである。

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ケ酸等の不溶性固形物を遠心分離等の適切な手段で分離除去し、残留液中に含まれているペプチド混合物を乾燥等により回収する。

次に、このペプチド混合物を水等に溶解させた状態で膜分離、イオン交換分離、ゲル濾過分離等により分離精製し、それを更に高速液体クロマトグラフィー（例えば逆相カラムを用いた高速液体クロマトグラフィー等）等により処理して上記よりペプチドを純粋な状態で回収する。

上記したペプチド混合物を含有する水溶液の分離精製およびトリペプチドの単離は、例えば次の(a)~(f)の工程からなる方法で行うことができる。

(a) ペプチド混合物を含有する水溶液のpHを約3.0~5.0に調整し、これをイオン交換クロマトグラフィーにかけ（例えば東ソー株式会社製のSP-Toyopearl 550Cを充填したカラムに通過させる）、このクロマトグラフィーに吸着した成分を0.0Mから0.5Mまでの塩酸濃度

る。

プロテアーゼ活性の測定法

高価として米国ノルック社製のハマー・スライン・カゼイン1%溶液を用い、アンソニー・法（「食品分析」第2巻、第237頁（昭和30年1月10日、財団法人発行））により測定した。反応は30℃で30分間行い、1分間に1μgのクロシン相当量を遊離するのに要する酵素量を1 unitとした。

プロテアーゼ活性は、各々の状況（例えばプロテアーゼの種類、プロテアーゼの使用量、基質濃度、処理時間等の条件を調整して行うのがよく、例えば上で挙げたプロテアーゼを比較する場合にはpH約1.5~5.0、温度約30~50℃で、0.75Mトリクロロ酢酸への分解率が約40~70%になるまで加水分解を行うとよい。

目的とする加水分解状態が達成された時点で加熱および/またはpH調整してプロテアーゼを失活させ、失活した酵素、未分解乳タンパク

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質を含有するNaCl水溶液で処理し、得られる各成分の中から高い阻害活性を有する成分（NaCl水溶液濃度が約0.1~0.5Mの範囲で溶解してくる成分）を回収する。

(b) 上記高い阻害活性を有する成分と分子ふるい処理して（例えばバイオラッド社製のバイオゲルP-2を充填したカラムを通過させる）、更にいくつかの成分に遊離水で抽出分離してその中から更に高い阻害活性を有する成分を回収する。

(c) 上記(b)で回収した成分を高速液体クロマトグラフィー（例えば東ソー株式会社製のODS-120T）に通過させ、吸着成分を0.1%トリフルオロ酢酸水溶液（A液）とアセトニトリルを50%含有する0.1%トリフルオロ酢酸水溶液（B液）との混合液であって混合液中のB液の濃度が0%から100%まで段階的に増加する逆相濃度勾配法を用いて分離すると、アセトニトリルの濃度が約20~22%の範囲の濃度範囲に高阻害ピークが現れ、この

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細分のACE阻害性を測定確認して選択する。

- (d) 必要に応じて上記(c)の工程を繰返す。(e)
(d)工程で得られた成分から高純度を電透析により除去して白色の固体を回収し、そして
(f) 上記白色固体として得られた生成物のアミノ酸配列を例えば液体相作製の無相式プロテインシーケンサー(PSQ-1システム)等を用いて調べ、Leu-Lys-Proからなるトリペプチドであることを確認する。

また、本発明のトリペプチドを化学合成により製造する場合は、例えば次の方法を採用することができる。

本発明のトリペプチドの化学合成法

ペプチド合成装置(ファルマシア社(スウェーデン)製のBiosynth 4170)を使用して合成する。具体的には、ポリアミド樹脂にFmoc-プロリンを結合させた後そのFmoc基を除去して保護アミノ基を露出させ、この露出アミノ基にFmoc-リジンを反応させてからFmoc基を除去し、更

にFmoc-ロイシンを反応してからFmoc基を除去して上置樹脂で保護されたペプチドを形成する。これを95%トリフルオロ酢酸水溶液と窒素で60分間反応させて樹脂を分解させた後、樹脂を除去する。トリフルオロ酢酸不溶成分を残渣除去した後、残留物を0.1N塩酸に溶解し、その溶液を高感度ゲルクロマトグラフィー(GDS-120T)に通して不純物を除去することによって純度の高いLeu-Lys-Proを単離する。

本発明のACE阻害剤は人間および他の動物に投与することができる。少量の投与によって顕著な血圧降下および上昇抑制を達成することができる。

本発明のACE阻害剤の許容な投与量は、投与される人間や動物の年齢、体重、性別、病状、動物の個体差の種々の条件によって異なる。

そして、本発明のACE阻害剤は経口投与および経鼻投与のいずれによっても投与可能であり、更に経皮で投与しても、また製薬工業において通常使用されている固形剤や液状剤

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と一併に投与してもよく、又は他の薬剤と混合または組合わせて使用することができる。また投与形態は、錠剤、丸剤、顆粒剤、カプセル、散剤、水溶液、注射剤等の任意の形態が可能である。

更に、本発明のACE阻害剤は、食品や飼料中に添加して、またはそれらと一緒に投与することもでき、その場合には天然タンパク質に由来するL-Leu-L-Lys-L-Proが望ましい。

以下に、本発明を例を挙げて具体的に説明するが本発明はそれらによって限定されない。

実施例

乳清タンパク質(日本プロテイン株式会社ALACEN 132)5gを0.03N塩酸100mlに分散溶解させた後、蒸留水を加えて全量200mlにした。1N塩酸を加えてpHを2.0に調整した後、ペプシン(末田シグマ薬業)5000unitsを加え、37℃で15時間反応させた。次に、5N水酸化ナトリウム水溶液でpHを4.4に調整した後、アスベールメス産のアルバルチックプロテイナー

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ゼ(大野薬品工業のプロテアーゼM)1000unitsを加えて45℃で5時間反応させた。次いで、5N水酸化ナトリウム水溶液でpHを6.0に調整した後90℃で20分間加熱して酵素を失活させるとともに未反応物を沈殿させた。室温に冷却した後、10000Gで20分間遠心分離して懸濁物を分離除去した。上清液を回収して凍干処理してペプチド配合率4.0gを得た。

上記で得たペプチド配合物500mgを5ml酢酸緩衝液50mlに溶解した後、1N塩酸でpH3.5に調整した。

これを流速15ml、長さ200mmのカラムにシリカゲル(株式会社特のSP-Tsuyopon)550Gを40mg充填したイオン交換クロマトカラムに1.0ml/分の流速で通過させた後、このクロマトカラムに吸着した成分を0Mから0.5Mまでの塩酸濃度勾配を有するNaCl水溶液からなる希釈液120mlを1ml/分の流速で流してカラムから回収したところ、NaCl水溶液濃度が0.4~0.5Mの成分に高い阻害活性を示す成分を得たのでこれを固

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収した。

次いで、上記画分をバイオラッド社の画分のバイオゲル P-2 を 200nm 宛切したカラム(カラム長さ 10cm、孔径 1000nm) に 0.33nm/分の流速で通して分子ふるい処理し、次に蒸留水で希釈してその中から高い阻害活性を有する画分を回収した。

上記画分をアッセイ試薬仕製の高速液体クロマトグラフィー-005-120T に 1nm/分の流速で通過させた後、検出成分を 0.1% トリフルオロ酢酸水溶液 (A 液) とアセトニトリルを 50% 含有する 0.1% トリフルオロ酢酸水溶液 (B 液) との混合液であって混合液中の B 液の濃度が 0% から 100% まで段階的に増加する阻害濃度調整液を 1nm/分の流速で通して希釈すると、アセトニトリルの濃度が 20~22% の阻害画分が高い阻害活性を有していたのでこの画分を回収し、この高速液体クロマトグラフィー処理を再度実施した。

得られた画分から画分を乾燥除去して白色の

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粉末を得る。次に 3 N 塩酸 200nm 加え、蒸留水で 50 倍に希釈する。約 30 分後に分光光度計で測定波長 300nm、透光波長 490nm における透光強度 (A) を測定する。試料試の代わりに阻害剤 50nm を同様に処理して透光強度 (B) を測定する。

阻害率は $B - A / B$ により算出される。

試料液の濃度を覚えて、阻害活性を上記と同様に測定し、活性を 50% 阻害する濃度を求めてこれを IC₅₀ として算した。

[ペプチドの ACE 阻害活性 (IC₅₀)]

ペプチド	IC ₅₀ (nm)
D-L-Leu-L-Lys-L-Pro-OH (本発明)	2.3
ブラジキニン・ポテンシエーター B	6.4
ブラジキニン・ポテンシエーター C	29.0

上記表の結果から、本発明の ACE 阻害剤は既知の ACE 阻害剤ブラジキニン・ポテンシエーター B および C に比べて極めて低濃度で、すなわちごく少量の使用で IC₅₀ を達成することができる。ACE 阻害活性が非常に高いことが

図 1(200nm) を回収した。この白色固体を本発明所製の蒸留式プロテインシーケンサー (P50-1 システム) を使用してそのアミノ酸配列を調べたところ、N 末端から順次 L-Leu、L-Lys および L-Pro が遊離してきた。このことから、D-L-Leu-L-Lys-L-Pro-OH で表されるトリペプチドであることが確認された。

上記で調製したトリペプチドおよび既知の ACE 阻害剤ペプチドの ACE 阻害活性を下記の方法で測定したところ、下記表に示す結果を得た。

ペプチドの ACE 阻害活性の測定法

試料液 50nm を試験管にとり、これに ACE 液 (単結晶性試薬のうきと純度の ACE の 1 unit を水 5 nm に溶解したもの) 20nm を加える。37℃ で 5 分間温めた後、亜硝酸 (SMB Bio-His-(601088.2)) を加えて 37℃ で 30 分反応させ、次いで 0.3 M 水酸化ナトリウム水溶液 1 nm を加えて反応を停止させる。次に試薬オルトファタルデヒド 100nm を加えて室温で 10 分間反応

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わかる。

[発明の効果]

本発明の ACE 阻害剤は、極めて少量の投与で ACE の活性を阻害して血圧低下および血圧上昇抑制を達成することができる。

また、本発明の ACE 阻害剤は、白色の水溶性粉末であるために、そのまままたは水等に溶解させて経口投与および経静脈投与のいずれの方法によっても極めて簡単に投与することができる。

その上、本発明の新規なトリペプチド Leu-Lys-Pro は、3 個のアミノ酸が配列しただけの極めて簡単な構造を有する低分子化合物であるため、化学合成によっても簡単に製造することができ、しかも投与した場合に体内での吸収性がよく高い血圧降下作用を示す。

特許出願人 日本製薬株式会社

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NEW PEPTIDE AND ANGIOTENSIN-CONVERTING ENZYME INHIBITOR

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NEW PEPTIDE AND ANGIOTENSIN-CONVERTING ENZYME INHIBITOR

[Shinkina peptide oyobi angiotensin henkan koso sogaizai]

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[There is no amendment in this translation]

Claim

1. Peptide and its salt expressed by the following equation.

Leu-Lys-Pro

2. Angiotensin-converting enzyme inhibitor having the peptide or its salt expressed by the following equation as the effective component.

Leu-Lys-Pro

Detailed explanation of the invention

Industrial application field

This invention concerns a new peptide and angiotensin-converting enzyme inhibitor having said peptide or its salt as the effective component.

Prior art

The renin-angiotensin system as a representative in vivo factor which results in raising blood pressure and the kallikrein-kinin system as a representative in vivo factor which functions to lower blood pressure are known, and the angiotensin-converting enzyme (will be referred to as "ACE" below) contributes greatly to both of these systems.

The mechanism will be briefly explained. First, in the renin-angiotensin system, the enzyme renin from the kidney which is secreted into the blood interacts with angiotensinogen in the blood, and forms angiotensin I, which is a decapeptide. This angiotensin I does not display the blood pressure raising action; however, when ACE interacts with this, it forms angiotensin II, which is an octapeptide. This angiotensin II contracts the peripheral blood vessels and also interacts with the adrenal cortex and promotes the production of aldosterone. Aldosterone interacts with the kidney and results in an increase in the heartbeat output rate through the invitation of the reabsorption of sodium and an increase in the amount of body fluid. Both of them significantly increase the blood pressure.

On the other hand, in the kallikrein kinin group, the kallikrein as the enzyme in the blood interacts with kininogen, which is a precursor protein in the blood, and frees and produces kinin. However, this kinin dilates the peripheral blood vessels and also activates phospholipase A₂, promotes the synthesis of prostaglandins, and lowers blood pressure. However when ACE interacts with this kallikrein-kinin group, ACE breaks down and inactivates the aforementioned kinin, which has the dilating action of the peripheral blood vessels and the activation action of phospholipase A₂, and lowering of blood pressure does not occur.

Accordingly, when a substance which inhibits the aforementioned action by ACE (ACE inhibitor) is present, the formation of angiotensin II, which is a blood pressure raising substance, is inhibited, and the breakdown of kinin, which functions as blood pressure lowering substance, is prevented as well, and the control over raising the blood pressure and lowering the blood pressure becomes possible.

From such a viewpoint, various types of research and development of ACE inhibitors have been implemented in recent years, and it has been reported that specific peptides that originate from natural proteins or through synthesis have the ACE inhibiting action. The ACE inhibitors originating from natural proteins that have been reported so far include bradykinin potentiator B (Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro) and bradykinin potentiator C

(Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro) that originate from the mamushi (both are described in H. Kato and T. Suzuki, *Biochemistry*, 10, p. 972 (1971)), milk casein originating peptides Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys (Japanese Kokoku Patent No. Sho 60[1985]-23085), Phe-Phe-Val-Ala-Pro (Japanese Kokai Patent No. Sho 59[1984]-44323), Thr-Thr-Met-Pro-Leu-Trp (Japanese Kokai Patent No. Hei 2[1990]-20263), Ala-Val-Pro-Tyr-Pro-Gln-Arg, fish protein originating peptides Tyr-Lys-Ser-Phe-Ile-Lys-Gly-Tyr-Pro-Val-Met and Pro-Glu-Glu-Glu-Pro-His-Val-Leu, corn γ -zein originating peptides Leu-Pro-Pro, Val-His-Leu-Pro-Pro, and Val-His-Leu-Pro-Pro-Pro, for example. The majority of these are peptides in which 5 or more amino acids are linked together.

Content of the invention

Under the aforementioned circumstances, the inventors of this invention also have advanced the research on the substances having the ACE inhibiting action.

As a result, it has been discovered that a new tripeptide Leu-Lys-Pro, in which leucine-lysine-proline are arranged with an amino acid sequence different from the aforementioned existing ACE inhibiting peptide, can be isolated from a hydrolyzed milk serum protein, and this tripeptide has the ACE inhibiting action.

Accordingly, this invention is a peptide and its salts expressed by the following equation.

Leu-Lys-Pro

This invention also includes the ACE inhibitor with the peptide or its salt expressed by the aforementioned equation as the effective component.

The peptide having the aforementioned ACE inhibiting action in this invention was first discovered as a product through a hydrolysis treatment by the protease of a milk serum protein, in which all of the aforementioned 3 kinds of amino acids Leu, Lys, and Pro are L-amino acids in that case. However, without being limited only to these, any optical isomer can be used if it is a tripeptide having the aforementioned amino acid sequence, including tripeptides in which all of said 3 types of amino acids are made of D-amino acids, and tripeptides, in which any one or 2 of the 3 types of amino acids are L-amino acid and the rest are D-amino acid, and they can be manufactured by chemical synthesis.

An example of the preparation method of the tripeptide in this invention is listed below.

Method by the hydrolysis of a milk serum protein

A milk serum protein is hydrolyzed by using a protease, and a water-soluble milk serum protein originating peptide mixture was prepared. During this, a hydrolysis of the milk serum protein in a condition in which it was dispersed or dissolved in a liquid, such as water, for

example, is desirable from the viewpoints of easy operation, yield of the target product, and purity.

As the protease, a protease that reacts in acid is desirable, and, the use of an aspartic protease having the aspartic acid residue and the carboxylic acid ion of the aspartic acid related to the activity center of the enzyme is particularly desirable. Examples of such a protease include pepsin and orange polyporus-originating aspartic protease, *Aspergillus*-originating aspartic protease, and *Penicillium* originating aspartic protease. Pepsin and the *Aspergillus*-originating aspartic protease in particular are desirable at the point of obtaining the target product at a high yield. Only 1 kind of protease can be used, or several types can be used in combination as long as there is no negative effect extended among the proteases. When using several types of proteases, said several proteases can be simultaneously present and hydrolyzed, or 1 kind may be successively hydrolyzed at a time. The protease may be used in a free or stabilized state. The ideal amount of the protease that is used in all cases is about 5000-100,000 units per 100 g of dry glutene.

The protease activity (unit) in the specifications here is entirely measured by the method below.

Measurement method of the protease activity

Using a 1% Hammerstein casein solution manufactured by US Melk Co. as the matrix, it was measured by the Anthon-Hagiwara [transliteration] Modified Method (Edited by Shiro Akabori, "Enzymatic Research Method," Vol. 2, Page. 237 (published on January 10, 1961 from Asakura Books)). The reaction was held at 30°C for 30 min, and the amount of enzyme required for isolating tyrosine in an amount equivalent to 1 μ g in 1 min was established as 1 unit.

It is desirable to select the conditions for the optimal pH, temperature, protease amount, processing speed, and the processing time, etc., of the protease process according to various circumstances (for example, the type of protease, the form of protease used, etc.). When using the protease listed above, for example, a hydrolysis may be performed at a pH of about 1.5-5.0 and a temperature of about 30-50°C until the dissolution ratio into a 0.75M trichloroacetic acid reaches about 40-70%.

At the point of reaching the target state of hydrolysis, the protease was inactivated by heating and/or adjusting the pH, the inactivated enzyme and insoluble solids, such as the milk serum protein that had not decomposed, for example, were separated and eliminated by a proper measure, such as centrifugal separation, for example, and a peptide mixture contained in the residue solution was collected through drying, for example.

Successively, this peptide mixture in a condition in which it is dissolved in water, for example, was separated and purified through a membrane fraction, ion-exchange fraction, and a

gel filtration fraction, for example. This was furthermore processed through high-speed liquid chromatography (high-speed liquid chromatography using a reverse-phase column, for example), for example, and the aforementioned tripeptide was isolated in a pure form.

The separation and purification of an aqueous solution containing the aforementioned peptide mixture and the isolation of a tripeptide can be obtained by a method consisting of (a)-(f) processes below.

(a) The pH of the water solution containing the peptide mixture was adjusted to about 3.0-5.0; this was run through ion-exchange chromatography (passing through a column packed with SP-Toyoperal 550 C manufactured by Toso K.K., for example), the component which had adsorbed onto this chromatography was eluted by a aqueous NaCl solution having a linear concentration gradient from 0M to 0.5M, and fractions having a high inhibition activity (fractions in which the concentration of the NaCl water solution was eluted within a range of about 0.4-0.5M) were collected from the fractions that were obtained.

(b) The aforementioned fractions having a high inhibition activity were processed by a molecular filtration (passing through a column packed with Biogal P-2 manufactured by BioLad Co., for example), and were furthermore eluted and separated into several fractions by distilled water, and fractions having a higher inhibition activity were collected from them.

(c) The fractions collected by the aforementioned (b) were passed through a high-speed liquid chromatography (ODS-120T manufactured by Toso K.K., for example), the components which were adsorbed were eluted by a linear concentration gradient eluent, which was a mixed solution of a 0.1% aqueous trifluoroacetic acid solution (solution A) and a 0.1% aqueous trifluoroacetic acid solution containing 50% acetonitrile (solution B), in which the concentration of solution B in the mixed solution increases linearly from 0% to 100%, a high adsorption peak appeared in the section of the eluent within a range of about 20~22% of the concentration of acetonitrile, and the ACE inhibition activity of this fraction was measured and confirmed, and collected.

(d) The aforementioned process (c) was repeated if necessary.

(e) The solvent was eliminated through drying, for example, from the fraction obtained through process (d), a white solid content was collected, and

(f) The amino acid sequence of the product obtained as the aforementioned white solid was checked by using a gas-phase protein sequencer (PSQ-I system) manufactured by Shimazu Seisakusho, for example, and the tripeptide consisting of Leu-Lys-Pro was confirmed.

Also, the following method can also be adopted, for example, when manufacturing the tripeptide in this invention through a chemical synthesis.

Chemical synthesis method of the tripeptide in this invention

It was synthesized using a peptide synthesis device (Biolynx 4170 manufactured by Pharmacia Co (Sweden)). Concretely, after condensing the Fmoc-proline with a polyamide resin, that Fmoc radical was removed, the terminal amino acid was isolated, the Fmoc-lysine was condensed with this free amino acid; then, the Fmoc radical was eliminated, and Fmoc-leucine was also condensed, the Fmoc radical was eliminated, and a peptide protected by the aforementioned resin was formed. This was reacted with a 95% aqueous trifluoroacetic acid solution at room temperature for 60 minutes and the resin was separated, and the resin was eliminated through filtering. After eliminating the trifluoroacetic acid water solution under reduced pressure, the residue was dissolved in 0.1N acetic acid, that solution was passed through a high-speed liquid chromatography (ODS-120T), impurities were eliminated, and Leu-Lys-Pro at a high purity was isolated.

The ACE inhibitor in this invention can be administered to humans and various types of animals, and a significant lowering of blood pressure and a control of its raising can be attained through a dosage in a small amount.

The satisfactory dosage of the ACE inhibitor in this invention is different according to various types of conditions, such as the age, weight, sex, and conditions of the humans and animals and the type of animal, etc., for the administration.

Then, the ACE inhibitor in this invention can be administered through both oral and nonoral administrations. Furthermore, it can be administered independently, or may be administered together with a solid support and a liquid support which are generally used in the pharmaceutical industry. Or, it can be used mixed together or combined together with other substances. Also, possible administration forms include any optional forms including tablets, round tablets, granulars, capsules, dispersing agents, aqueous solutions, and injection agents, etc.

Furthermore, the ACE inhibitor in this invention may be added into foods and feeds or can be administered together with them, and L-Leu-L-Lys-L-Pro which originates from a natural protein is ideal in that case.

This invention will be concretely explained in an example below; however, this invention should not be limited by it.

Application example

After dispersing and dissolving 5 g of milk serum protein (ALACEN 132 by Nippon Protein K.K.) in 100 mL of a 0.03N hydrochloric acid, distilled water was added to a total volume of 200 mL. After adjusting the pH to 2.0 by adding 1N hydrochloric acid, 5000 units of pepsin (manufactured by US Sigma Co.) were added, and reacted at 37°C for 15 h. Next, after adjusting the pH to 4.4 by the 5N aqueous sodium hydroxide solution, 1000 units of alpartic

[transliteration] protease originating from *Aspergillus* (Protease M manufactured by Amano Pharmaceutical Co.) were added, then reacted at 45°C for 5 h. Successively, after adjusting the pH to 6.0 with the 5N aqueous sodium hydroxide solution, [the solution was] heated to 90°C for 20 min and the enzyme was inactivated, and the undissolved substances were precipitated. After cooling to room temperature, solid contents were separated and removed through a centrifugal separation of 10,000 G for 20 min. The supernatant was collected, freeze-dried, and 4.0 g of a peptide mixture were obtained.

500 mg of the aforementioned peptide mixture obtained were dissolved into 50 mL of a 5mM acetic acid buffer solution; then, the pH was adjusted to 3.5 by the 1N hydrochloric acid.

This was passed through an ion-exchange chromatocolumn packed with 40 mL SP-Toyopearl 550C manufactured by Toso K.K. in a column with a diameter of 16 mm and a length of 200 mm at a flow rate of 1.0 mL/min, the components that were adsorbed onto this chromatocolumn were eluted from the column by running 120 mL of an eluate consisting of the aqueous NaCl solution having a linear concentration gradient of 0M to 0.5M at a flow rate of 1 mL/min, fractions having a high inhibition activity in the area of 0.4-0.5M in the concentration of the aqueous NaCl solution were obtained, and they were collected.

Successively, the aforementioned fractions were passed through a column (diameter of the column is 16 mm, and the length is 1000 mm) packed with 200 mL of Biogal P-2 manufactured by BioLad Co. at a flow rate of 0.33 mL/min, processed by a molecular filtration, eluted with distilled water next, and fractions having a high inhibition activity were collected from them.

After passing the aforementioned fractions through a high-speed liquid chromatograph ODS-120T manufactured by Toso K.K. at a flow rate of 1 mL/min, the components that were adsorbed were eluted by running an eluate, which was a mixed solution of a 0.1% aqueous trifluoroacetic acid solution (solution A) and a 0.1% aqueous trifluoroacetic solution containing 50% acetonitrile (solution B), and has a linear concentration gradient in which the concentration of solution B in the mixed solution increased linearly from 0% to 100%, at a flow rate of 1 mL/min, the concentration of the acetonitrile had a high inhibition activity in the section of the eluate of 20-22%, these fractions were collected, and this high-speed liquid chromatography processing was repeated again.

The solvent was dried and eliminated from the fractions that were obtained, and 1200 µg of a white solid content were obtained. The amino acid sequence of this white solid content was checked by using a vapor phase type protein sequencer manufactured by Shimazu Seisakusho (PSQ-1 system), L-Leu, L-Lys, and L-Pro successively isolated from the N terminal. Through this, the tripeptide expressed by equation $\text{H}\cdot\text{L-Leu-L-Lys-L-Pro}\cdot\text{OH}$ was confirmed.

The ACE inhibition activity of the tripeptide prepared above and the existing ACE inhibiting peptides were measured by the method below, and the results indicated in the table below were obtained.

Measurement method of the ACE inhibiting activity of peptides

50 μ L of the sample solution were put in a test tube, 20 μ L of the ACE solution (1 unit of ACE originating from a rabbit lung manufactured by US Sigma Co. was dissolved in 5 mL water) were added to this. After maintaining this at 37°C for 5 min, the substrate (5 mM Hip-His-Leu: pH 8.3) was added, and reacted at 37°C for 30 min. Successively, 1 mL of a 0.3M aqueous sodium hydroxide solution was added, and the reaction was stopped. 100 μ L of a fluorescent test drug orthophthalaldehyde solution was added, and reacted at room temperature for 10 min. Next, 200 mL of 3N hydrochloric acid were added, and diluted 50 times with distilled water. The fluorescent intensity (A) at the excitation wavelength of 300 nm and the fluorescent wavelength of 490 nm were measured by a spectrofluorophotometer after about 30 min. 50 μ L of distilled water were similarly processed instead of the sample solution, and the fluorescent intensity (B) was measured.

The inhibition activity can be obtained by B-A/B.

Changing the concentration of the sample solution, the inhibition activity was measured in the same aforementioned manner, the concentration for inhibiting 50% of the activity was obtained and this was expressed as $1C_{50}$.

① [ペプチドのACE阻害活性 ($1C_{50}$)]	
② ペプチド	$1C_{50}(\mu M)$
H-L-Leu-L-Lys-L-Pro-OH (本発明) ③	2.2
ブラジキニン・ポテンシエーターB ④	6.4
ブラジキニン・ポテンシエーターC ⑤	29.0

- Key: 1 (The ACE inhibition activity ($1C_{50}$) of peptide)
 2 Peptide
 3 (This invention)
 4 Bradykinin potentiator B
 5 Bradykinin potentiator C

From the results in the aforementioned table, it can be understood that the ACE inhibitor in this invention is a solution with a very low concentration when compared to the existing ACE inhibitors bradykinin potentiators B and C, in other words, IC_{50} can be attained in a very small amount of use, and the ACE inhibition activity is very high.

Effect of the invention

The ACE inhibitor in this invention inhibits the ACE activity and attains a lowering of the blood pressure and a control of the raising of the blood pressure through its administration in a very small amount.

The ACE inhibitor in this invention is also in the form of a white water-soluble powder; therefore, it can be very easily administered either through oral administration or nonoral administration directly or while being dissolved in water, for example.

Moreover, the new tripeptide Leu-Lys-Pro in this invention is a low-molecular-weight compound having a very simple structure where only 3 amino acids are arranged, which can be easily manufactured through a chemical synthesis as well. Moreover, it displays an excellent adsorptivity in the body and a high-blood-pressure lowering action when administered.

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(71)Applicant: Nisshin Flour Product
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(74)Patent attorney: Chika Takagi

1.Title:New Peptide and Angiotensin converting enzyme-inhibitor

2. Range of Patent petition

1) Peptide Leu-Lys-Pro and its salt

2) Angiotensin converting enzyme-inhibitor from peptide Leu-Lys-Pro and its salt.

3. Description of invention

(Extent of commercial use)

This invention is about angiotensin converting enzyme-inhibitor from new peptide Leu-Lyn-Pro, existing one, and their salt.

(Former technique)

Renin-angiotensin system (r-a) is one of the factors of hypertension. Kallikrein-kinin system (k-k) is one of the factors of antihypertension. Angiotensin converting enzyme (ACE) takes part in both of them. In r-r in blood, renin from kidney produces angiotensinI, decapeptide, with angiotensinagen. When angiotensinI reacts with ACE, it produces angiotensinII that contracts peripheral blood vessel and promotes aldosterone from adrenal cortex. Aldosterone makes kidney reabsorb sodium and increase body fluid to increase blood pressure.

On the other hand, in k-k, kininogen reacts kallikrein to release kinin that expands peripheral blood vessel, activates phospholipaseA2, and accelerates synthesis of prostaglandin to reduce blood pressure.

However, when ACE reacts to r-a, ACE breaks and deactivates kinin. Therefore, if ACE inhibitor exists, it is possible to suppress producing angiotensinII and breaking kinin.

Recently some researches have indicated special peptides made from nature and synthesis inhibit ACE.

Natural ACE inhibiting peptides:

-From pit viper: bradykinin potentiater B (Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro)

bradykinin potentiater C (Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro)

(H.Kato and T.suzuki, Biochemistry, 10, P.972, 1971)

-From milk casein: Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys(patent pub.#6023085)

Phe-Phe-Val-Ala-Pro(patent pub.#5944323)

Thr-Thr-Met-Pro-Leu-Trp(patent pub.#0220263)

Ala-Val-Pro-Tyr-Pro-Gln-Arg

-From fish protein: Tyr-Lys-Ser-Phe-Ile-Lys-Gly-Tyr-Pro-Val-Met

Pro-Glu-Glu-Glu-Pro-His-Val-Leu

-From corn γ -zein: Leu-Pro-Pro

Val-His-Leu-Pro-Pro

Val-His-Leu-Pro-Pro-Pro

Most of the peptides are more than 5 chains of amino acid.

(Content of the invention)

As the result of the inventor's research, he has invented that he can extract new peptide from hydrolyzed whey protein that inhibits ACE.

This invention is the amino acid sequence, Leu-Lys-Pro, and its salt.

This invention includes ACE inhibitor.

The ACE inhibiting peptide is found after hydrolysis of whey protein with proteinase. These 3 amino acids are all L-Amino acids, but optical isomer is not matter if tripeptide that has all 3 amino acid is used.

These amino acids can be even produced by synthesis from tripeptide of D-amino acid that has one or two L-amino acid.

The materials for hydrolyzing whey protein

-Whey protein solution w/distilled water

-Proteinase for acid condition

Aspartic proteinase: from pepsin; Aspergillus; Penicillium.

Best if use one from pepsin or Aspergillus.

The proteinase can be mixed with others before and after adding to the whey solution.

The condition of the proteinase is either free or fixed.

The concentration of the proteinase 5,000~100,000 units per 100g gluten powder.

The method of measuring proteinase activity

-Use 1% solution of hammar stain casein (Melk, U.S.)

-Use Anson-Hagiwara Method (S. Akahori, "Enzyme Research Method", No.2, p. 237, 1/10/1961, Asakura Book)

-1 unit=the amount of enzyme to extract 1 μ g of tyrosine per 1min. at 30C for 30 min.

The method of hydrolysis

1. pH about 1.5~5.0; temp. about 30~50C.

2. Hydrolyzing until 40~70% of the solubility of 0.75M trichloroacetic acid.

3. Use heat or pH adjust to deactivate the proteinase, and centrifuge it to remove undissolved whey protein.

4. Dry the peptide solution, and collect the powder.

5. Reconstitute the powder.

6. Purify the tripeptide by using membrane, ion exchange, gel filtration, and extract the tripeptide with a reverse column of HPLC.

a. Adjust pH 3.0~5.0, and adsorb tripeptide with ion exchange chromatography (resin: Sp-Toyopearl 550C).

b. Desorb the component by using NaCl solution from 0M to 0.5M as linear gradient, and collect high ACE inhibitor which comes out when NaCl solution is 0.4M~0.5M.

c. Purify the solution with Bio-Rad Biogel P-2 column and distilled water.

d. Use HPLC(ODS-120T, Tosoh, Inc.) to absorb the solution, and desorb the peptide with mixture of 0.1% trifluoroacetic acid (A) and 0.1% trifluoroacetic acid that of 50% is acetonitrile (B).

e. Use linear gradient method that the amount of (B) increases gradually from 0% to 100%, and there is high concentration of ACE inhibiting activity when the concentration of acetonitrile is 20 to 22%.

f. Repeat above procedure as many as it needs, and dry it.

g. Use protein sequencer (PSQ-I system, Shimadzu Manufacture) to confirm that tripeptide consist of Leu-Lys-Pro.

The method of tripeptide synthesis

-Use peptide synthesis instrument (Biolyne 4170, Falmashia, Sweden).

-Condense Fmoc-proline on polyamide resin and remove Fmoc group from it.

-Condense Fmoc-lysine on free amino group, and remove Fmoc group.

-Condense Fmoc-leucine on it, and remove Fmoc group.

-Remove the resin from the peptide by using 95% trifluoroacetic acid at room temp, and remove trifluoroacetic acid by vacuum.

-Mix with 0.1N acetic acid, and extract Leu-Lys-Pro with HPLC(ODS-120T).

Conclusion

This ACE inhibitor can be used small amount for humans and animals to decrease blood pressure and prevent hypertension. The amount of it is depend on age, weight, gender, symptom, and kind of animal and humans. It is possible to take it orally or not and to mix with liquid or solid carrier or without. It can be mixed with other medicines, and can be form of tablets, powder, capsules, injections. Natural ACE inhibitor, L-Leu-L-Lys-L-Pro, can be added to food and animal feed.

Example

- Dissolve 5g of whey protein (ALACEN 132,Nihon Protein, Inc.) into 100ml of 0.03N HCl, and add distilled water up to 200ml.
- Adjust pH 2.0 with 1N HCl, and react with 5000units of pepsin (Sigma) at 37C for 15 hours.
- Adjust pH 4.4 with 5N NaOH, and react with 1000 units of Alpatic Proteinase (ProteinaseM, Amano Pharmaceutical, Inc.) at45C for 5 hours.
- Adjust pH 6.0 with 5N NaOH, and heat at 90C for 20 min. to deactivate enzyme and precipitate non-dissolve materials.
- Cool down the liquid temp., and centrifuge it at 10000G for 20 min. to remove solid materials.
- Collect the supernatant, and freeze dry to get 4.0g of powder.
- Dissolve 500mg of the powder into 50ml of 5mM acetic acid buffer, and adjust pH 3.5 with 1N HCl.
- Run this solution into the column, 16mmX200mm, with 40ml of SP-Toyopearl 550C (Toso, Inc.) at 1.0ml per min. of flow, and run 120ml of NaCl solution by using liner gradient method from 0M to 0.5M at 1ml per min. of flow.
- Collect high fraction of ACE inhibitor at 0.4~0.5M NaOH, and use the column, 16mmX1000mm, with 200ml of Biogel P-2 (Bio-Rad) at 0,33ml per min. of flow to filter.
- Run distilled water to collect eluent.
- Use HPLC(ODS-120T, Toso, Inc.) to absorb the solution, and desorb the peptide with mixture of 0.1% trifluoroacetic acid (A)and 0.1% trifluoroacetic acid that of 50% is acetonitrile (B).
- Use liner gradient method that the amount of (B) increases gradually from 0% to 100%, and there is high concentration of ACE inhibiting activity when the concentration of acetonitrile is 20 to 22%.
- Repeat above procedure, and dry it.
- Collect 1200µg of powder, and use protein sequencer (PSQ-1system, Shimazu Manufacture) to confirm that tripeptide consist of H•L-Leu-L-Lys-L-Pro•OH.

The method of ACE inhibiting activity for the peptide

- Mix 50µl of the peptide solution with 20µl of ACE solution (mix 1 unit of ACE from rabbit lung of Sigma with 5ml of distilled water), and heat at 37C for 5 min.
- Add the substrate (5mM Hip-His-Leu : pH8.3), and heat at 37C for 30 min., and add 1ml of 0.3M NaOH to stop the reaction.
- Add 100µl of orthophthalate aldehyde, fluorescent tester, and react at room temp. for 10 min.
- Add 200ml of 3N HCl, and dilute 50 times with distilled water.
- After 30 min., measure the fluorescent intensity of the solution (A) at 300µm of excited wave and 490µm of fluorescent wave, and the fluorescent intensity of control sample without the peptide is (B).
- Inhibiting activity formula: B-A/B
- Adjust the activity to 50% by changing the concentration of the peptide.

(ACE inhibiting activity (IC50))

Peptide	IC50(µM)
H•L-Leu-L-Lys-L-Pro•OH	2.2
bradykinin potentiater B	6.4
bradykinin potentiater C	29.0

-The result shows that the small amount of the ACE inhibiting peptide invented can reach to IC50 that means high ACE inhibiting activity.

(Effect of invention)

- This ACE inhibitor can be used small amount to decrease blood pressure and prevent hypertension. It is easy to take it orally or not, because this white powder can be dissolve in water, etc.
- ACE inhibitor, Leu-Lys-Pro, is simple structure, 3-amino acid chain; therefore, it is also easy to produce this peptide by synthesis and to absorb into body.

Applicant: Nisshin Flour Product

Patent attorney: Chika Takagi